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The *TFG-TEC* fusion gene created by the t(3;9) translocation in human extraskeletal myxoid chondrosarcomas encodes a more potent transcriptional activator than *TEC*

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The t(3;9)(q11–q12;q22) translocation associated with human extraskeletal myxoid chondrosarcomas results in a chimeric molecule in which the N-terminal domain (NTD) of the *TFG* (TRK-fused gene) is fused to the *TEC* (Translocated in Extraskeletal Chondrosarcoma) gene. Little is known about the biological function of *TFG-TEC*. Because the NTDs of TFG-TEC and TEC are structurally different, and the TFG itself is a cytoplasmic protein, the functional consequences of this fusion in extraskeletal myxoid chondrosarcomas were examined. The results showed that the chimeric gene encoded a nuclear protein that bound DNA with the same sequence specificity as the parental TEC protein. Comparison of the transactivation properties of TFG-TEC and TEC indicated that the former has higher transactivation activity for a known target reporter containing TEC-binding sites. Additional reporter assays for TFG (NTD) showed that the TGF (NTD) of TFG–TEC induced a 12-fold increase in the activation of luciferase from a reporter plasmid containing GAL4 binding sites when fused to the DNA-binding domain of GAL4, indicating that the TFG (NTD) of the TFG–TEC protein has intrinsic transcriptional activation properties. Finally, deletion analysis of the functional domains of TFG (NTD) indicated that the PB1 (Phox and Bem1p) and SPYGQ-rich region of TFG (NTD) were capable of activating transcription and that full integrity of TFG (NTD) was necessary for full transactivation. These results suggest that the oncogenic effect of the t(3;9) translocation may be due to the TFG-TEC chimeric protein and that fusion of the TFG (NTD) to the TEC protein produces a gain-of-function chimeric product.

Introduction

Chromosome translocation is a process by which a piece of one chromosome is detached and moved to another. It is one of the main mechanisms that creates oncogenes and generates human cancers (1). Human extraskeletal myxoid chondrosarcomas (EMCs) are soft tissue tumors characterized by specific chromosomal abnormalities. These diseases have an uncertain histogenetic origin and arise primarily within the musculature, most commonly the thigh and knee (2–4). Most cases of human EMCs harbor characteristic translocations, t(9;22)(q22;q12) or t(9;17)(q22;q11.2), which involve the *TEC* (Translocated in Extraskeletal Chondrosarcoma) gene at 9q22 and the *EWS* (Ewing's sarcoma) gene at 22q12 or the *hTAF*$_{II}$*68* (human TATA-binding protein-associated factor II 68) gene at 17q11.2 (5,6). Recently, a proportion of EMCs were found to harbor a characteristic translocation, t(3;9)(q11–q12;q22), involving the *TFG* (TRK-fused gene) at 3q11–q12 and the *TEC* gene at 9q22 (7).

The *TFG* was originally identified as a fusion partner of the *NTRK1* gene in human papillary thyroid carcinoma (8) and is also involved in another chromosome translocation with the *ALK* gene in anaplastic large-cell lymphomas (9). It is located on the q arm of human chromosome 3 and encodes a ubiquitously expressed cytoplasmic protein (8). The total length of *TFG* cDNA is 1677 bp, encoding a 400 aa protein containing putative functional domains, such as Phox and Bem1p (PB1), a coiled-coil (CC) domain and a serine, proline, tyrosine, glycine and glutamine (SPYGQ)-rich region (10). Interestingly, the TFG protein interacts with the NF-κB Essential Modulator and TRAF family member-associated NF-κB activator proteins, suggesting that it may play a key role in NF-κB regulation (11). However, the function of *TFG* in normal cells is not clear.

The *TEC* gene is the human homologue of the rat *NOR-1* receptor (12) and encodes a novel orphan nuclear receptor belonging to the steroid/thyroid receptor gene superfamily (2,4). It is also called *CHN* (2) and *MINOR* (13) and is located at 9q22 (14). *TEC* was originally identified as a fusion partner of the *EWS* gene in human EMCs (4). This gene spans ~40 kb and has eight exons, of which exons 1 and 2 correspond to the 5′-untranslated sequence of the mature *TEC* mRNA (14). Although the biological role of *TEC* remains undefined, constitutive expression of *TEC* leads to marked cell death in thymocytes (15), suggesting that it may be implicated in cell proliferation, possibly by controlling downstream target genes.

Therefore, to investigate how *TFG-TEC* functions as an aberrant transcription factor that contributes to tumorigenesis in human EMCs, we analyzed its biochemical properties and transcriptional activation behavior. We found that it is a nuclear protein that binds DNA with a sequence specificity indistinguishable from that of the parental TEC protein. However, although TFG-TEC contains the complete amino acid sequence for TEC, it shows greatly increased transcriptional activation potential, indicating that the N-terminal portion of TFG-TEC may provide additional transactivation properties. Consistent with this, we demonstrated that the TFG (NTD) region serves as a transcription activation domain when coupled to a GAL4 DNA-binding domain (DBD). These data indicate that *TFG-TEC* may play a critical role in the formation of human EMCs by modulating the transcription of specific TEC target genes required for tumorigenesis.

Materials and methods

Materials and general methods

Restriction endonucleases, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from New England Biolabs. PfuTurbo polymerase was purchased from Stratagene, and [γ-^{32}P] ATP (3000 Ci/mmol) was obtained from PerkinElmer. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformations and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins were carried out according to standard methods (16). Subclones generated from polymerase chain reaction (PCR) products were sequenced using the chain termination method with double-stranded DNA templates to ensure the absence of mutations.

Constructs

pCMV-Tag2A/TFG-TEC was generated by amplifying the respective domains (TFG NTD and TEC) using PCR followed by splicing of the regions using the overlap extension method as described previously (17,18). Details regarding the construction of pCMV-Tag2A/TEC have been previously reported (18). The TFG-TEC reporter plasmid p(B1a)8-Luc has also been described previously (3).

To construct pCMV-Tag2A/TFG (NTD), TFG (NTD) was amplified from pCMV-Tag2A/TFG-TEC by PCR using the primers 5′-TFGBamHI (5′-GATCGGATCCAATGAACGGACAGTTGGA-3′; BamHI site underlined) and 3′-TFGHindIII (5′-GATCAAGCTTCTGAATACTGAATACCAT-3′; HindIII site underlined), digested with BamHI and HindIII and cloned into the same sites of the pCMV-Tag2A vector (Stratagene). To generate pCMV-Tag2A/TFG-TEC (AAAA), pCMV-Tag2A/TEC was digested with AccI and EcoRI and cloned into the corresponding sites of pBluescript II KS+

Abbreviations: CC, coiled-coil; DBD, DNA-binding domain; EMCs, extraskeletal myxoid chondrosarcomas; EMSAs, electrophoretic mobility shift assays; GST, glutathione S-transferase; NBRE, NGFI-B response element; NTD, N-terminal domain; PB1, Phox and Bem1p; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEC, Translocated in Extraskeletal Chondrosarcoma; TFG, TRK-fused gene.

(Stratagene) to yield pKSII/(AccI-EcoRI). To generate pKSII/(AccI-EcoRI) (AAAA), in which the amino acids KRRR were substituted with AAAA, we used the QuikChange™ site-directed mutagenesis kit (Stratagene) and the mutagenic primer set 5′-mNLS (5′-CTGCCCAGTAGACGCGGCAGCTGCAAACCGATGTCAG-3′; mutation sites underlined) and 3′-mNLS (5′-CTGACATCGGTTTGCAGCTGCCGCGTCTACTGGGCAG-3′; mutation sites underlined). pKSII/(AccI-EcoRI) (AAAA) was digested with AccI and EcoRI to isolate the (AccI-EcoRI) (AAAA) fragment, which was then cloned into the corresponding sites of pCMV Tag2A/TEC to generate pCMV Tag2A/TEC (AAAA). Then, pCMV Tag2A/TEC (AAAA) was digested with XhoI to isolate the XhoI(AAAA)XhoI fragment, which was cloned into the corresponding site of pCMV-Tag2A/TFG-TEC to generate pCMV-Tag2A/TFG-TEC (AAAA).

To construct pGEX (4T-1)-EWS (NTD), an EWS (NTD) fragment was amplified from pcDNA3/Flag-EWS-TEC by PCR using the primers 5′-EcoRIEWS1 (5′-GATCGAATTCATGGCGTCCACGGATTAC-3′; EcoRI site underlined) and 3′-NotIEWS265 (5′- GATCGCGGCCGCTCAACTCTGCTGCCCGTA-3′; NotI site underlined). The PCR product was digested with EcoRI and NotI and cloned into the same sites of the pGEX (4T-1) vector (GE Healthcare) to generate pGEX (4T-1)-EWS (NTD). The construct pGEX (4T-1)-hTAF$_{II}$68 (NTD) has been described previously (19). To construct pGEX (4T-1)-TFG (NTD), a TFG (NTD) fragment was amplified from pCMV-Tag2A/TFG-TEC by PCR using primers 5′-BamHITFG1 (5′-GATCGGATCCATGAACGGACAGTTGGAT-3′; BamHI site underlined) and 3′-NotITFG274 (5′-GATCGCGGCCGCTCAATCTGAATACTGAATACC-3′; NotI site underlined). The PCR product was digested with BamHI and NotI and cloned into the same sites of pGEX (4T-1) to generate pGEX (4T-1)-TFG (NTD).

To generate pcDNA3-EGFP, an EGFP gene was PCR amplified from pEGFP-N1 (Clontech) using primers 5-HindIIIEGFP (5′-GATCAAGCTTATGGTGAGCAAGGGCGAG-3′; HindIII site underlined) and 3′-BamHIEGFP (5′-GATCGGATCCGCTTACTTGTACAGCTCGTC-3′; BamHI site underlined). The PCR product was digested with HindIII and BamHI and cloned into the same sites in pcDNA3 to generate pcDNA3-EGFP. To construct pcDNA3-EGFP-TEC, an EGFP (no Stop) fragment was PCR amplified from pEGFP-N1 using primers 5′-HindIIIEGFP and 3′-BamHIEGFP(NoStop) (5′-GATCGGATCCGCCTTGTACAGCTCGTC-3′; BamHI site underlined). The PCR product was digested with HindIII and BamHI and cloned into the same sites in pcDNA3 to generate pcDNA3-EGFP(NoStop). pcDNA4/HisMaxB/TEC was digested with BamHI to isolate the TEC fragment, which was then cloned into the corresponding site in pcDNA3-EGFP(NoStop) to generate pcDNA3-EGFP-TEC. pcDNA3-EGFP-TFG-TEC was generated as follows: pCMV-Tag2A/TFG-TEC was digested with BamHI to isolate the TFG-TEC fragment, which was then cloned into the corresponding site in pcDNA3-EGFP(NoStop) to generate pcDNA3-EGFP-TFG-TEC.

The GAL4-TFG (NTD) deletion mutants were generated as follows. (A) GAL4-TFG (NTD): the TFG (NTD) fragment was amplified from pCMV-Tag2A/TFG-TEC by PCR using the primers 5′-BamHITFG_GAL4 (5′-GATCGGATCCGAATGAACGGACAGTTGG-3′; BamHI site underlined) and 3′-HindIIITFG_GAL4 (5′-GATCAAGCTTCTGAATACTGAATACCATAC-3′; HindIII site underlined), digested with BamHI and HindIII and cloned into the corresponding sites of the pM vector (Clontech Laboratories) to generate GAL4-TFG (NTD). (B) GAL4-TFG (1-124): the TFG (1-124) fragment was amplified from pCMV-Tag2A/TFG-TEC by PCR using the primers 5′-BamHITFG_GAL4 and 3′-HindIIITFGCC_GAL4 (5′-GATCAAGCTTTTCCAAGCTATCCAATAA-3′; HindIII site underlined), digested with BamHI and HindIII and cloned into the corresponding sites of the pM vector to generate GAL4-TFG (1-124). (C) GAL4-TFG (97-273): the TFG (97-273) fragment was amplified from pCMV-Tag2A/TFG-TEC by PCR using the primers 5′-BamHITFGCC_GAL4 (5′-GATCGGATCCGACTTGAATCAAGTCAGGTG-3′; BamHI site underlined) and 3′-HindIIITFG_GAL4, digested with BamHI and HindIII and cloned into the corresponding sites of the pM vector to generate GAL4-TFG (97-273). (D) GAL4-TFG (PB): the TFG (PB) fragment was amplified from pCMV-Tag2A/TFG-TEC by PCR using the primers 5′-BamHITFG_GAL4 and 3′-HindIIITFGPB_GAL4 (5′-GATCAAGCTTGGGTCTTGGCTGGCCATT-3′; HindIII site underlined), digested with BamHI and HindIII and cloned into the corresponding sites of the pM vector to generate GAL4-TFG (PB). (E) GAL4-TFG (CC): the TFG (CC) fragment was amplified from pCMV-Tag2A/TFG-TEC by PCR using the primers 5′-BamHITFGCC_GAL4 and 3′-HindIIITFGCC_GAL4, digested with BamHI and HindIII and cloned into the corresponding sites of the pM vector to generate GAL4-TFG (CC). (F) GAL4-TFG (SPYGQ): the TFG (SPYGQ) fragment was amplified from pCMV-Tag2A/TFG-TEC by PCR using the primers 5′-BamHITFGSPYGQ_GAL4 (5′-GATCGGATCCGACCACCTGGAGAACCAGGAC-3′; BamHI site underlined) and 3′-HindIIITFG_GAL4, digested with BamHI and HindIII and cloned into the corresponding sites of the pM vector to generate GAL4-TFG (SPYGQ).

In vitro transcription and translation

In vitro transcription and translation of Flag-TFG-TEC and Flag-TEC was carried out using the TNT kit (Promega) and pCMV-Tag2A/TFG-TEC or pCMV-Tag2A/TEC, respectively, according to the manufacturer's instructions (Promega). *In vitro* translation products were subjected to electrophoresis on 8% SDS-PAGE gels and analyzed by western blotting with an anti-Flag antibody (Sigma). Quantitation of *in vitro* translated proteins was performed using the ChemiDoc™ XRS System (Bio-Rad).

Electrophoretic mobility shift assay

The sequences of the synthetic oligonucleotide probes used in the electrophoretic mobility shift assays (EMSAs) were described previously (3). Probes (0.5 ng each) were prepared by end-labeling annealed complementary oligonucleotides with [γ-^{32}P] ATP using T4 polynucleotide kinase. DNA-binding reactions were performed with *in vitro* translated TFG-TEC and TEC for 30 min at 4°C in binding buffer containing 10 mM Tris–HCl (pH 8.0), 40 mM KCl, 6% glycerol, 1 mM DTT, 0.05% NP-40 and 10 ng/μl of poly (dI·dC)·(dI·dC). Following the binding reaction, the mixtures were separated on 4% polyacrylamide gels (acrylamide/bisacrylamide ratio, 37:1) in 0.5× TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA) buffer at 150 V for 2 to 3 h at 4°C. The gels were dried and exposed to Kodak X-Omat film at −70°C with an intensifying screen.

Subcellular localization

Immunocytochemical analyses were performed as described previously (20). Briefly, 293T cells were plated on glass coverslips and transfected with the respective DNA plasmids using the VivaMagic Reagent (Vivagen Co., Ltd). After 48 h, the cells were washed in phosphate-buffered saline and fixed for 10 min at −20°C in a mixture of acetone and methanol (1:1, v/v). Anti-Flag antibody (M2, Sigma) and a TRITC-conjugated secondary antibody (Sigma) were used to detect the Flag-tagged TFG-TEC or TFG-TEC mutant proteins, and fluorescence was observed under a fluorescence microscope (Olympus, IX71) equipped with a CoolSNAP digital camera (Olympus).

Purification of GST fusion proteins, GST pull-down assays and western blot analysis

Glutathione *S*-transferase (GST)-EWS (NTD), GST-hTAF$_{II}$68 (NTD) and GST-TGF (NTD) proteins were expressed in *Escherichia coli* as described previously (21). After binding to Glutathione-Sepharose (GE Healthcare) and washing, the proteins were eluted with reduced glutathione (Sigma). Protein concentrations were determined using the Bradford reagent (Bio-Rad). The purity and size of the eluted proteins were evaluated by Coomassie blue staining of SDS-PAGE gels. GST pull-down assays were performed as described previously (20). Western blot analysis was performed using anti-Flag (M2) or anti-Xpress (Invitrogen) antibodies, and reactive bands were detected by chemiluminescence using Western Lightning (PerkinElmer Life Sciences).

Reporter gene assays

Cells were transiently transfected with plasmids using the VivaMagic Reagent, and luciferase assays were performed using the Dual-luciferase Assay System (Promega). Renilla luciferase activity was used to normalize the transfection efficiency.

Reverse transcription–polymerase chain reaction

Total RNA was prepared from 293T cells transiently transfected with EGFP, EGFP-TEC or EGFP-TFG-TEC using an RNeasy mini kit (Qiagen) with on-column DNase treatment, and messenger RNA was purified with an Oligodex-dT mRNA mini kit (Qiagen) followed by cDNA synthesis using a Superscript First-strand Synthesis System for reverse transcription–polymerase chain reaction (RT–PCR) (Invitrogen). RT-PCR reactions for *Skp2, L-Myc, SOCS2* and *STAT-3* genes were performed in triplicate with gene-specific primer sets. The following primers were used: Skp2 forward, 5′-CTGTCTCAGTGTTCCAAGTTGCA-3′, and Skp2 reverse, 5′-CAGAACACCCAGAAAGGTTAAGT-3′ (22); L-Myc forward, 5′-AGCGACTCGGAGAATGAAGA-3′, and L-Myc reverse, 5′-CAGCTTCTGGAGGAAAACG-3′ (23); SOCS2 forward, 5′-CTCGGTCAGACAGGATGGTA-3′, and SOCS2 reverse, 5′-ACAGAGATGCTGCAGAGATG-3′ (24); STAT-3 forward, 5′-TTGCCAGTTGTGGTGATC-3′, and STAT-3 reverse, 5′-AGAACCCAGAAGGAGAAGC-3′ (25) and β-actin forward, 5′-GCTCGTCGTCGACAACGGCTC-3′, and β-actin reverse, 5′-CAAACATGATCTGGGTCATCTTCTC-3′.

Results

TFG-TEC binds to a TEC consensus sequence

Human EMCs share recurrent translocations that fuse the EWS gene from 22q12 or the *hTAF$_{II}$68* gene from 17q11.2 with the *TEC*

nuclear receptor gene from 9q22. A recent report showed that one of the chromosomal translocation events in human EMCs results in the in-frame fusion of *TFG* on chromosome 3q11–q12 with *TEC* showing translocation t(3;9)(q11–q12;q22) (7). The breakpoint in *TFG* is in intron 6, and the breakpoint in *TEC* is two nucleotides (A and T) upstream of the ATG initiation codon. TFG is a cytoplasmic protein, which contains PB1 and CC domains at the N-terminus and SPYGQ-rich transcriptional activator-like domains at the N- and C-termini (8,10). The predicted chimeric protein comprises the N-terminal PB1, CC and SPYGQ-rich domains of TFG fused with full-length TEC (Figure 1).

The DNA-binding properties of the TFG-TEC protein were analyzed using EMSA incorporating a synthetic oligonucleotide probe and an *in vitro* transcribed/translated TFG-TEC protein. The DBD of TEC is a conserved DBD that binds to the NGFI-B Response Element (NBRE) sequence motif (5′-AAAAGGTCA-3′) (3). Although there is considerable structural variation between TFG-TEC and TEC, the DBD of TEC is intact in both proteins. To determine whether TFG-TEC binds to the physiological targets of TEC, an EMSA was performed using the NBRE sequence motif as a target in the binding reaction. Synthetic RNAs produced by *in vitro* transcription of full-length Flag-tagged *TFG-TEC* and *TEC* were used to program cell-free rabbit reticulocyte lysates, and the resultant *in vitro*–translated Flag-tagged TFG-TEC and Flag-tagged TEC proteins were quantified by SDS-PAGE and western blotting with an anti-Flag antibody (M2) (Figure 2A). Equimolar amounts of *in vitro* translated TFG-TEC and TEC were added to each assay. Quantitation of *in vitro* translated proteins was performed using the ChemiDoc™ XRS System (Bio-Rad). EMSAs were performed using a single concentration of probe and increasing amounts of *in vitro*–translated protein. Protein–DNA complexes were formed in the presence of both TFG-TEC (Figure 2B, lanes 8–10) and TEC (Figure 2B, lanes 5–7), whereas unprogrammed reticulocyte lysate showed a very low level of binding (Figure 2B, lanes 2–4). The interaction was specific as the complexes were disrupted by a 5- and 10-fold excess of unlabeled oligonucleotide containing the NBRE sequence motif, but not by an oligonucleotide containing a mutated NBRE sequence motif not recognized by the TEC DBD (Figure 2C). We concluded from these experiments that TFG-TEC and TEC display similar DNA-binding activity *in vitro* and bind DNA with similar specificity.

The KRRR sequence in the DBD targets TFG-TEC to the nucleus

Since TFG is a cytoplasmic protein (8) and TFG-TEC contains the NTD of TFG fused to full-length TEC, we determined the subcellular localization of TFG-TEC by indirect immunofluorescence. Indirect immunofluorescence analysis was performed using 293T cells because TFG-TEC-positive human EMC cell lines, or their derivatives, are not currently available. 293T cells were transfected with an empty expression vector (pCMV-Tag2A, data not shown) or pCMV-Tag2A/TFG-TEC [Figure 3B(a)] and analyzed by fluorescence immunohistochemistry. Chimeric TFG-TEC localized to the nucleus [Figure 3B(a)], indicating that TFG-TEC is a nuclear protein. Next, a set of TFG-TEC deletion mutants was used to map the region of TFG-TEC responsible for nuclear localization (Figure 3A). 293T cells were transfected with expression vectors for Flag-tagged TFG-TEC truncation mutants, and the localization of Flag-tagged proteins was analyzed by fluorescence immunohistochemistry. Flag-tagged TFG (NTD) localized to the cytoplasm [Figure 3B(b)], whereas Flag-tagged TEC clearly localized to the nucleus [Figure 3B(c)]. To further define the nuclear localization signal in the DBD of TFG-TEC, we generated a DBD mutant in which several highly conserved basic amino acids, ^{612}KRRR^{615}, were replaced with alanine residues using site-directed mutagenesis. Substitution of ^{612}KRRR^{615} with alanines resulted in the cytoplasmic accumulation of Flag-tagged TFG-TEC [Figure 3B(d)]. This result suggests that this cluster of basic amino acids in the DBD functions as a nuclear localization signal for TFG-TEC.

The TFG (NTD) is defective for U1C binding

The EWS (NTD) contains interaction motif(s) for a splicing factor, U1C. The interaction between EWS (NTD) and U1C negatively regulates EWS-fusion-mediated transactivation (26). Because of the relatedness of the EWS and TFG NTDs (7,10), we assessed whether

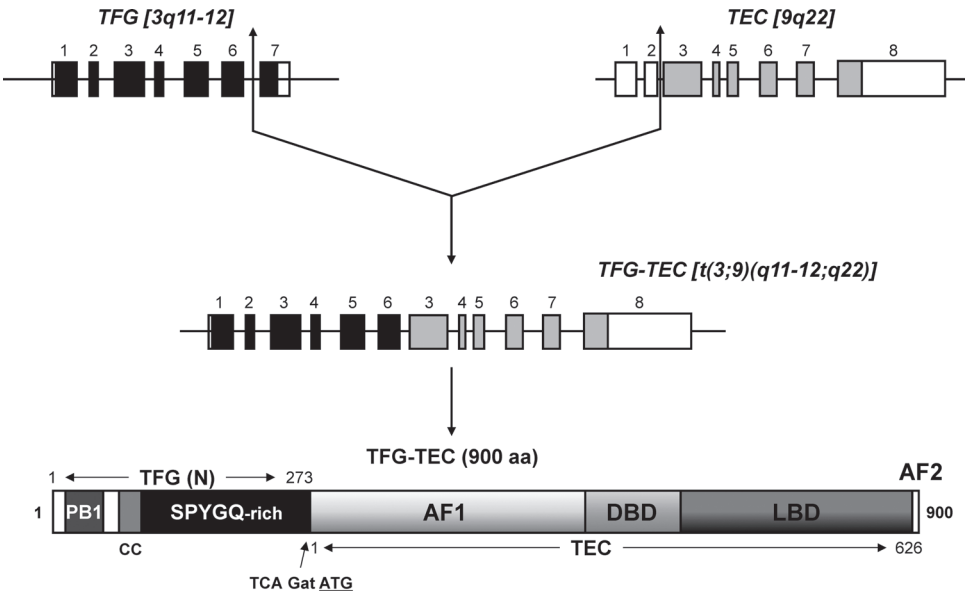

Fig. 1. Schematic representation of the TFG-TEC fusion in human extraskeletal myxoid chondrosarcoma. The exon/intron structures of the *TFG* and *TEC* genes are shown. The exons are indicated by boxes, and the coding regions of the *TFG* and *TEC* genes are represented by black and shaded boxes, respectively. Open boxes represent the non-coding regions of the genes. Numbers refer to exons of the *TFG* and *TEC* genes. Amino acid position is also indicated above or below the schematic representing the TFG-TEC fusion protein. The first 273 aa [residues 1–273; TFG (N)] of *TFG* are fused to residues 1–626 of *TEC* via one additional amino acid (aspartic acid) encoded by the combination of one nucleotide (G) from exon 6 of *TFG* and two nucleotides (A and T) from the 5′ UTR of *TEC* in *TFG-TEC*. Functionally important domains within the TFG-TEC chimera are indicated: PB1, Phox and Bem1p domain; CC, coiled-coil domain; SPYGQ-rich, Ser, Pro, Tyr, Gly, Gln-rich domain; AF1, N-terminal transactivation domain; DBD, DNA-binding domain; LBD, ligand-binding domain and AF2, C-terminal transactivation domain. The two nucleotides upstream of the ATG initiation codon of *TEC* are represented by small letters, and the ATG initiation codon of *TEC* is indicated by underlined capital letters in TFG-TEC.

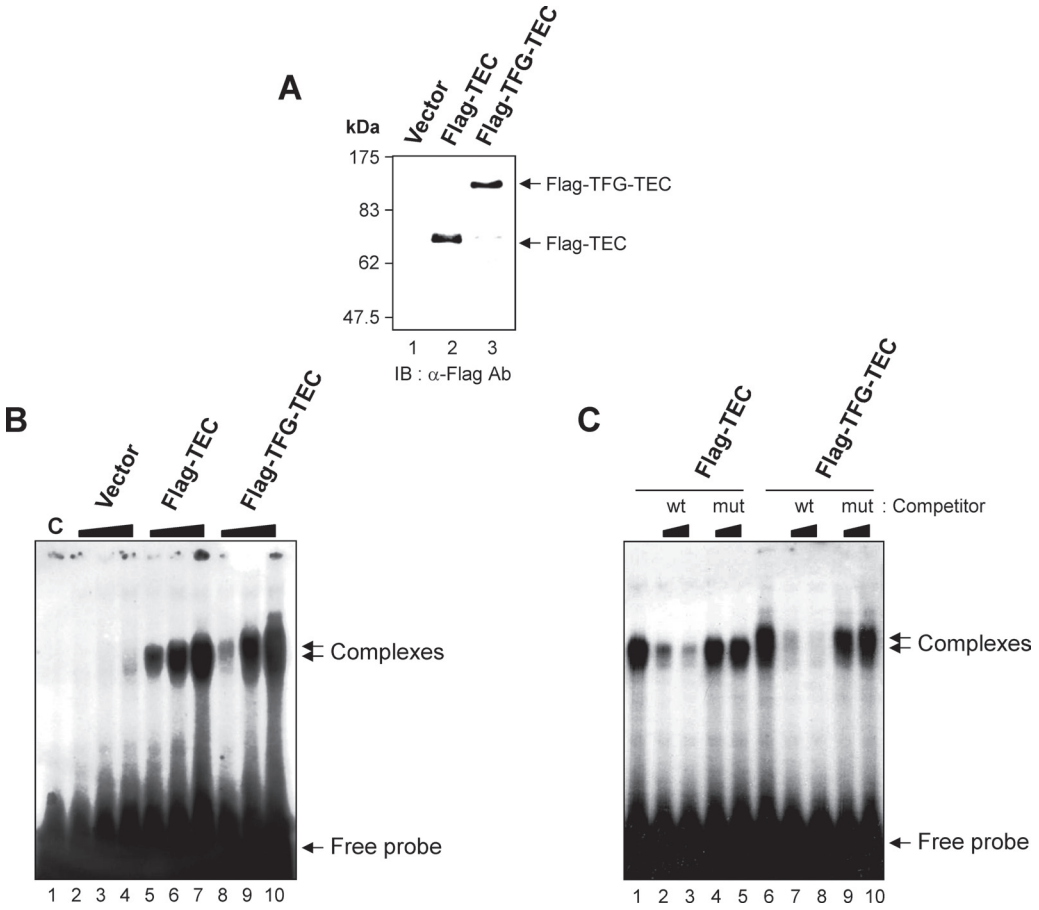

Fig. 2. Sequence-specific DNA binding by the TFG-TEC chimera. (**A**) Western blot analysis of *in vitro*–translated TFG-TEC and TEC proteins. Rabbit reticulocyte lysates were programmed with vector mRNA (lane 1), Flag-TEC mRNA (lane 2) or Flag-TFG-TEC mRNA (lane 3). Five microliters of the translation reaction product were resolved on 8% SDS-PAGE gels and analyzed by western blotting with an anti-Flag antibody (M2). Migration of the prestained molecular weight markers (New England Biolabs) is indicated on the left. (**B**) EMSAs of the DNA-binding properties of TFG-TEC and TEC. EMSAs were performed using either unprogrammed reticulocyte lysate (lane 1), reticulocyte lysate programmed with vector (lane 2, 2 µl; lane 3, 6 µl; lane 4, 10 µl), reticulocyte lysate programmed with Flag-TEC mRNA (lane 5, 2 µl; lane 6, 6 µl; lane 7, 10 µl) or reticulocyte lysate programmed with Flag-TFG-TEC mRNA (lane 8, 2 µl; lane 9, 6 µl; lane 10; 10 µl) and a radiolabeled probe as described in Materials and methods. The *in vitro*–translated proteins used in each EMSA are indicated above the gel. Protein-DNA complexes were resolved on non-denaturing 4% polyacrylamide (acrylamide:bisacrylamide ratio, 37:1) gels run at 4°C in 0.5× TBE (44.5 mM Tris–HCl, 44.5 mM boric acid, 1 mM EDTA). The positions of free probe and the protein-DNA complexes are indicated. (**C**) Sequence-specific DNA binding by TFG-TEC. Competition experiments were performed with 5-fold (lanes 2 and 7) or 10-fold (lanes 3 and 8) excess of unlabeled wild-type TEC oligonucleotide or with 5-fold (lanes 4 and 9) or 10-fold (lanes 5 and 10) excess of an unlabeled mutated TEC oligonucleotide. The positions of free probe and the protein-DNA complexes are indicated by arrows.

TFG could also interact with U1C. To test this, *in vitro* GST pull-down assays were performed using bacterially expressed GST fusion EWS (NTD), $hTAF_{II}68$ (NTD) and TFG (NTD), and full-length U1C protein with a His_6 tag (Figure 4). As previously reported (26), U1C protein specifically bound to GST-EWS (NTD) (Figure 4A, lane 3) but not to GST alone (lane 2). Interestingly, U1C protein also interacted with another member of the TET family, $hTAF_{II}68$ (NTD) (lane 4), indicating that the EWS (NTD)/U1C interaction was faithfully maintained in the $hTAF_{II}68$ (NTD) protein. In contrast, TFG (NTD) did not associate with U1C (lane 5), suggesting that U1C is unable to modulate TFG-TEC activity in human EMCs. The amount of GST fusion protein utilized in these assays was fractionated on 15% SDS-PAGE gels and visualized by Coomassie blue staining. The results showed that similar amounts of protein had been used in the pull-down assays (Figure 4B).

TFG-TEC is a more potent transcriptional activator than TEC

The transcriptional properties of TFG-TEC and TEC were studied by cotransfecting the expression vectors with a reporter plasmid. The NTDs of EWS and $hTAF_{II}68$ appear to contribute to transcriptional activation by the EWS- or $hTAF_{II}68$-fusion proteins by providing a strong transactivation domain (27). To assess the transcriptional effects of NTD-TFG in TFG-TEC, we compared transcription by TFG-TEC and TEC by cotransfecting the respective expression vectors with a reporter plasmid containing eight copies of the TEC binding site (NBREs) and a TATA box upstream of the luciferase gene (Figure 5A). A control plasmid comprising a cytomegalovirus-driven Renilla luciferase gene was also included. Cotransfection of TFG-TEC induced a 635-fold increase in reporter expression in 293T cells (Figure 5B, bars 2 and 4) compared with the approximately 220-fold increase induced by TEC alone (Figure 5B, bars 1 and 3). Clearly, TFG-TEC is a much more potent transcriptional activator than TEC.

The finding that TFG-TEC had higher transactivation activity than TEC in the reporter assays was further investigated. To assess the transactivation potential of TFG-TEC *in vivo*, we transiently transfected 293T cells with the pcDNA3-EGFP, pcDNA3-EGFP-TEC and pcDNA3-EGFP-TFG-TEC constructs (Figure 5C) and compared the transcription activity of TFG-TEC and TEC with that of their potential downstream target genes, Skp2 (28), L-Myc, SOCS2 and STAT-3. As shown in Figure 5C, expression of Skp2, L-Myc, SOCS2 and STAT-3 was upregulated in 293T cells transfected with TFG-TEC to a greater extent than in 293T cells transfected with TEC (Figure

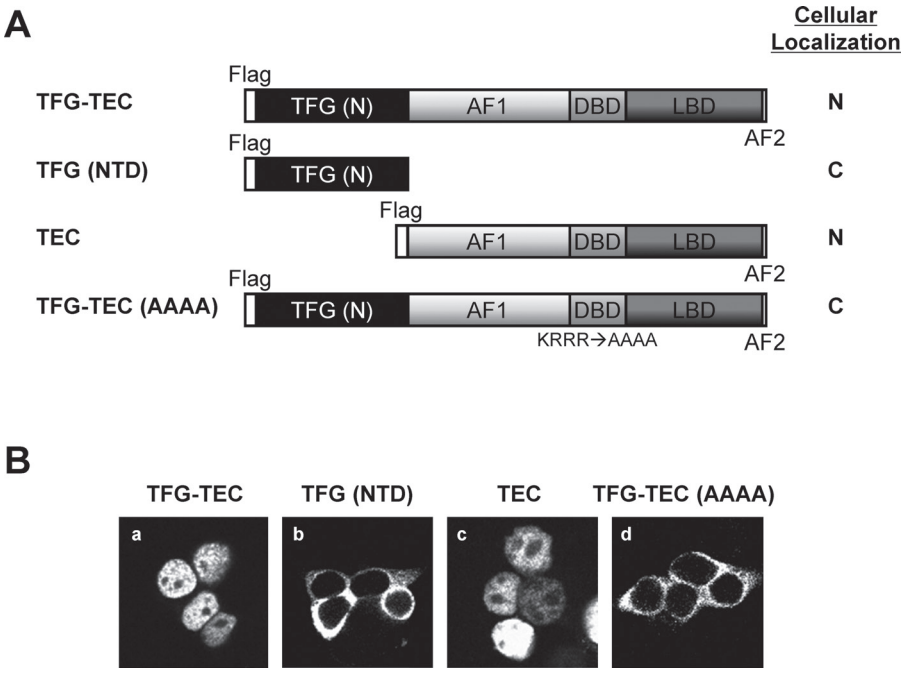

Fig. 3. Subcellular localization of TFG-TEC and mapping of the nuclear localization signal of TFG-TEC to the DNA-binding domain. (**A**) Schematic diagram of Flag-tagged TFG-TEC derivatives. Subcellular localization of the TFG-TEC, TFG (NTD), TEC and TFG-TEC (AAAA) constructs is indicated as N (nuclear localization) or C (cytoplasmic localization). (**B**) Subcellular distribution of TFG-TEC derivatives. 293T cells grown on coverslips were transfected with mammalian expression vectors encoding Flag-tagged TFG-TEC (a), TFG (NTD) (b), TEC (c) or TFG-TEC (AAAA) (d). Cells expressing Flag-tagged TFG-TEC derivative proteins were subjected to immunofluorescence microscopy with anti-Flag antibody (M2, Sigma).

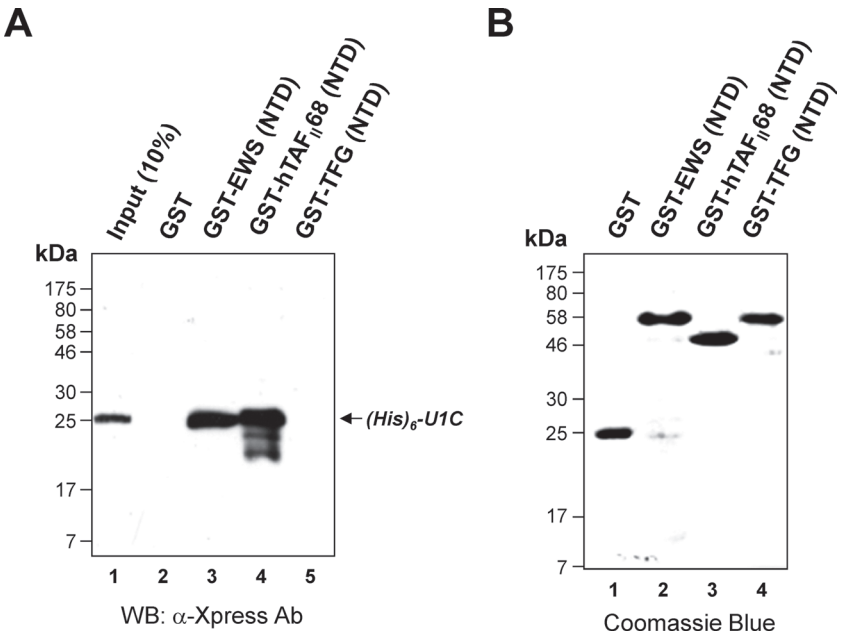

Fig. 4. Interactions between U1C and TFG (NTD). (**A**) Different binding affinities of EWS, hTAFII68 or TGF NTDs to U1C. GST pull-down experiments were performed essentially as described in Materials and methods. An aliquot of the input (10%) protein (lane 1), the pellet from the GST pull-down (lane 2), the pellet from the GST-EWS (NTD) (lane 3), the pellet from the GST-hTAF$_{II}$68 (NTD) (lane 4) and the pellet from the GST-TFG (NTD) (lane 5) were analyzed on 15% SDS-PAGE gels and bound U1C protein was detected using an anti-Xpress antibody (Invitrogen). The identities of the GST fusion proteins are indicated above the panel. The positions of the molecular mass markers are indicated on the left. U1C is indicated by an arrow on the right. Three independent experiments were performed, all of which gave similar results. (**B**) Quantitation of the GST fusion proteins used in the GST pull-down assays. The GST fusion proteins utilized in the pull-down assays were separated on 15% SDS-PAGE gels and visualized by Coomassie blue staining. Three independent experiments were performed, all of which gave similar results. Lane 1, GST alone; lane 2, GST fusion EWS (NTD); lane 3, GST fusion hTAF$_{II}$68 (NTD); lane 4, GST fusion TFG (NTD).

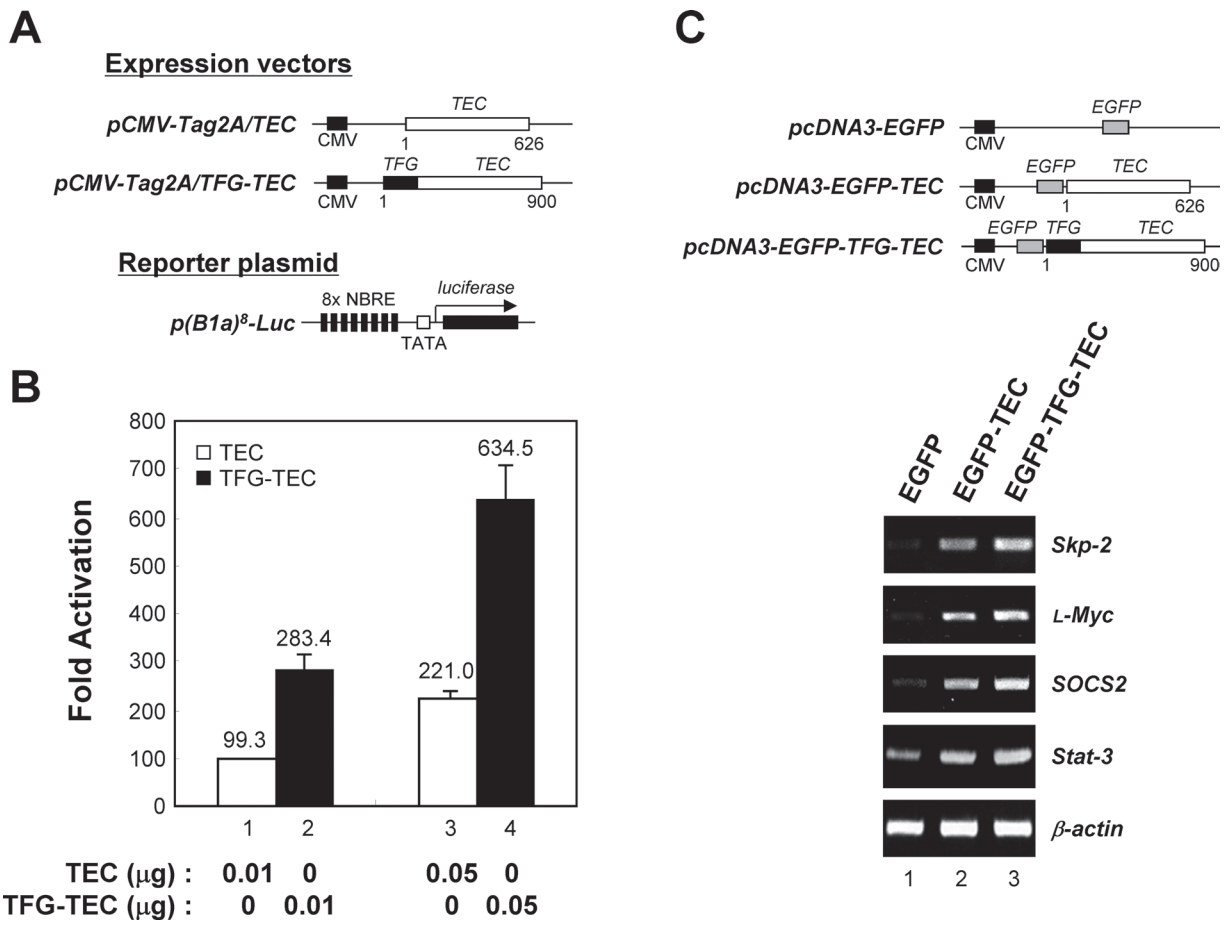

Fig. 5. Transactivation potential of TFG-TEC. (**A**) Schematic representation of the expression and reporter plasmids. The expression vectors driving the production of TFG-TEC or TEC are shown. The positions of the first and last amino acids are indicated below each construct. The p(B1a)^8-Luc reporter plasmid contains eight copies of the TEC recognition sites (NBRE) upstream of a basal promoter-luciferase construct. The eight copies are indicated by solid bars, the TATA box is represented by an open box and the luciferase gene is represented by a solid bar. (**B**) Transcriptional properties of TFG-TEC and TEC. 293T cells were cotransfected with expression vectors encoding the indicated amounts of TEC (white bars) or TFG-TEC (black bars), the p(B1a)^8-Luc reporter plasmid and the Renilla luciferase. Reporter activity was normalized with Renilla luciferase activity to correct for different transfection efficiencies. Fold induction is expressed relative to the empty expression vector. Each transfection was performed at least thrice independently, and the mean values are plotted with their standard errors (±SEM, vertical bars). (**C**) Induction of potential TEC downstream target genes by TFG-TEC *in vivo*. 293T cells were transiently transfected with pcDNA3-EGFP, pcDNA3-EGFP-TEC or pcDNA3-EGFP-TFG-TEC, and the transfected cells were isolated by FACS. RT–PCR analysis of Skp2, L-Myc, SOCS2 and STAT-3 mRNAs was performed in 293T cells expressing EGFP, EGFP-TEC or EGFP-TFG-TEC proteins. β-actin was used for normalization. Following amplification, an aliquot of each product was analyzed by staining the gel with ethidium bromide. The vectors pcDNA3-EGFP-TEC and pcDNA3-EGFP-TFG-TEC were used to express EGFP fused to TEC or TFG-TEC, respectively. The pcDNA3-EGFP expression vector was used as a control. The transiently transfected cell lines from which the input RNAs used in the RT reactions were derived are shown above the panel.

5C, lanes 2 and 3). Taken together, these data indicate that TFG-TEC is a more potent transcriptional activator than TEC both *in vitro* and *in vivo*. β-actin was used as the control.

TFG (NTD) functions as a transactivation domain

TFG-TEC was still a more potent transcriptional activator than TEC (Figure 5). Even though the TFG-TEC chimeric protein contained intact TEC functional domains (Figure 1), protein-DNA complexes were formed with both TFG-TEC and TEC proteins (Figure 4), and both were localized to the nucleus (Figure 3); this could be due to the differences in their respective NTDs. However, it remains unclear whether TFG (NTD) is able to activate transcription. Therefore, an expression vector containing a GAL4-TFG gene fusion was generated to assess the transcriptional effects of NTD-TFG (Figure 6A). The pG5 luc reporter, which contains five GAL4 DNA-binding sites upstream of the TATA box, was used as a reporter. The TFG (NTD) responsiveness of the pG5 luc reporter was evaluated by cotransfecting 293T cells with 0.1 μg of the pG5 luc construct and 2 ng of the Renilla plasmid together with increasing amounts of the GAL4-TFG (NTD) expression construct. An expression vector driving the synthesis of the GAL4 DBD alone (GAL4) had no significant effect on the level of luciferase produced by pG5 luc when transfected into 293T cells (Figure 6B, lane 1). In contrast, pGAL4-TFG (NTD) activated luciferase production in a dose-dependent manner (up to 12-fold; lanes 2–5). These results demonstrate that TFG (NTD) contains a transactivation domain(s).

Two functional domains are important for transactivation by TFG (NTD)

Transient transfection experiments with the GAL4-fusion functional domains of TFG (NTD) were performed to define the functional regions within TFG (NTD) required for transactivation. The structures of the TFG (NTD) functional domains are shown schematically in Figure 7. The data in the right panel show the relative transcriptional activation values, with the value obtained for GAL4-TFG (NTD) taken as 100%. Deletion of SPYGQ-rich region amino acids 125–273 [GAL4-TFG (1–124)] produced a polypeptide showing a 75% reduction in transactivation ability

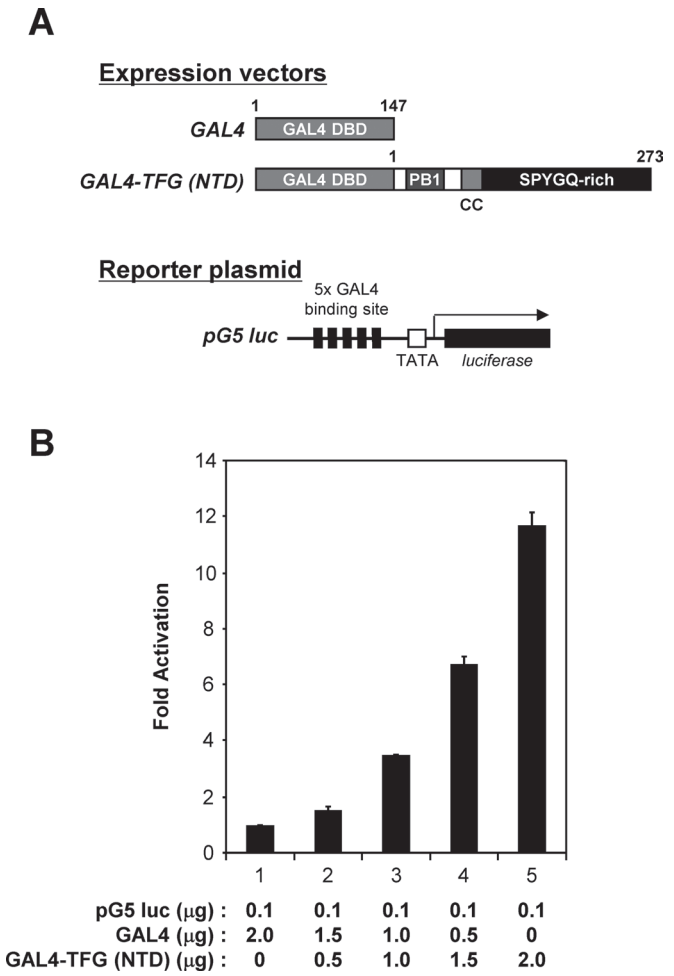

Fig. 6. Transactivation potential of TFG (NTD). (**A**) Schematic representation of GAL4-TFG (NTD) expression and the reporter plasmids used in this study. The expression vectors driving the production of the GAL4 DNA-binding domain (aa 1–147) and GAL4-TFG (NTD) are shown. The pG5 luc reporter plasmid contains five copies of GAL4-binding sites (5× GAL4-binding site) upstream of a basal promoter-luciferase construct. The five copies are indicated by solid bars, the TATA box is represented by an open box and the luciferase gene is represented by a solid bar. GAL4, GAL4 DNA-binding domain; TFG (NTD), TFG N-terminal domain of TFG-TEC; PB1, Phox and Bem1p domain; CC, coiled-coil domain; SPYGQ-rich, Ser, Pro, Tyr, Gly, Gln-rich domain. (**B**) Transcriptional activation by GAL4-TFG (NTD). 293T cells were transfected with 0.1 μg of pG5 luc reporter plasmid, 2 ng of Renilla luciferase and 0 μg (lane 1), 0.5 μg (lane 2), 1.0 μg (lane 3), 1.5 μg (lane 4) or 2.0 μg (lane 5) of GAL4-TFG (NTD) plasmid. In all experiments, the total amount of transfected DNA was adjusted with an empty vector (GAL4 DNA). Reporter activity was normalized with Renilla luciferase activity to correct for different transfection efficiencies. Luciferase activity was expressed as fold activation relative to the basal level observed with the reporter plasmid and the GAL4 DNA-binding domain alone (lane 1). Each transfection was performed independently at least thrice, and the mean values are plotted with their standard errors (±SEM, vertical bars).

relative to GAL4-TFG (NTD). Removal of the PB1 domain [GAL4-TFG (97–273)] resulted in a smaller reduction (about 50%) in transactivation. These results indicate that these domains are important for TFG (NTD)–mediated transactivation. The fusion of the SPYGQ-rich region [named GAL4-TFG (SPYGQ)] increased luciferase production from pG5 luc by 30%, indicating that the SPYGQ-rich region of TFG (NTD) has intrinsic transcriptional activation properties. In addition, the PB1 domain of TFG (NTD) also affected the level of luciferase (13%) produced from pG5 luc when transfected into 293T cells. On the other hand, CC produced a polypeptide with only 6% of the transactivation potential of the polypeptide produced by GAL4-TFG (NTD). We interpret these results to indicate that the PB1 domain and the SPYGQ-rich region of TFG (NTD) are partially capable of activating transcription and that full integrity of the TFG (NTD) is necessary for full transactivation activity.

Discussion

The *TFG* gene was originally identified through homology searches within the Expressed Sequence Tag Database using SPYGQ-rich regions as query sequences to identify genes encoding protein regions similar to the N-terminal portions of the EWS and TLS/FUS proteins (10). Although these amino acids are commonly found in transcriptional activation domains (27), the function of this region in TFG-TEC was still unknown. Therefore, to investigate the biological functions of this chimeric gene, we characterized the TFG-TEC fusion protein. In the present study, we show that TFG-TEC is a nuclear protein that binds DNA with sequence specificity indistinguishable from that of the parental TEC protein. This fusion gene encodes a transactivator that is more potent than TEC.

U1C, which functions as a splicing factor and is important during the early stages of spliceosome formation, associates with EWS-Fli-1 (26,29). This interaction is mediated via the NTD of EWS and modulates EWS-Fli-1 activity by inhibiting EWS-Fli-1-mediated transcriptional activation (26). Interestingly, the U1C/EWS (NTD) interaction is faithfully maintained in $hTAF_{II}68$ (NTD) (Figure 4). However, TFG (NTD) is defective for U1C binding (Figure 4), indicating that the spectrum of TFG (NTD) interaction partners might not be the same as that of the EWS (NTD)–centered protein interaction network. Thus, it would be interesting to test whether TFG-TEC is able to interact with known EWS (NTD)–interacting proteins, such as c-Abl (30), v-Src (31) and ZFM1 (32), and whether its activity is controlled by these proteins.

Unlike other fusion products, such as EWS-Fli-1 (33,34), EWS-WT1 (35) and EWS-Oct-4 (36,37), which can transform NIH3T3 cells or cause increased tumor growth in nude mice, the predicted transforming activity of TFG-TEC has yet to be determined. The tumorigenic potential of the TFG-TEC gene product is consistent with the idea that it plays a crucial role in the formation of human EMCs. Because the DNA-binding specificities of the TFG-TEC protein resembled those previously defined for the TEC (Figure 2) and the TFG-TEC gene encodes a strong transcriptional activator (Figure 5), it is conceivable that TFG-TEC fusion contributes to tumorigenesis by deregulating the expression of TEC-responsive genes. However, even though TFG-TEC fusion seems to play an oncogenic role via the inappropriate activation of TEC target genes equivalent to those of other TEC fusions in human EMCs, definitive identification of the critical target gene(s) is still required.

As shown in Figure 5C, ectopically expressed TFG-TEC and TEC upregulated the expression of Skp2, L-Myc, SOCS2 and STAT-3. In addition, we noted that these potential target genes were more strongly upregulated by TFG-TEC than by TEC (Figure 5C). NOR1 functions as a mitogen (38–40), but the mechanisms underlying this activity remain obscure. Recently, it was reported that NOR1 induces expression of the Skp2 gene (28), which is responsible for degrading the cyclin-dependent kinase inhibitor, p27 (41,42). Ectopically expressed TFG-TEC also upregulated the expression of L-Myc, SOCS2 and STAT-3. Interestingly, expression of L-Myc, SOCS2 and STAT-3 is also observed in developing human cancers (43–46). These findings seem to be important because they may explain how TFG-TEC plays an oncogenic role in the development of human extraskeletal myxoid chondrosarcoma.

Generation of a new chimeric transcription factor with enhanced functionality by chromosomal translocation is an invariant theme among EWS- and $hTAF_{II}68$-TEC fusion proteins in human EMCs (27). According to our previous report, the $hTAF_{II}68$ NTD of $hTAF_{II}68$-TEC contributes to its function by providing a novel activation domain (47),

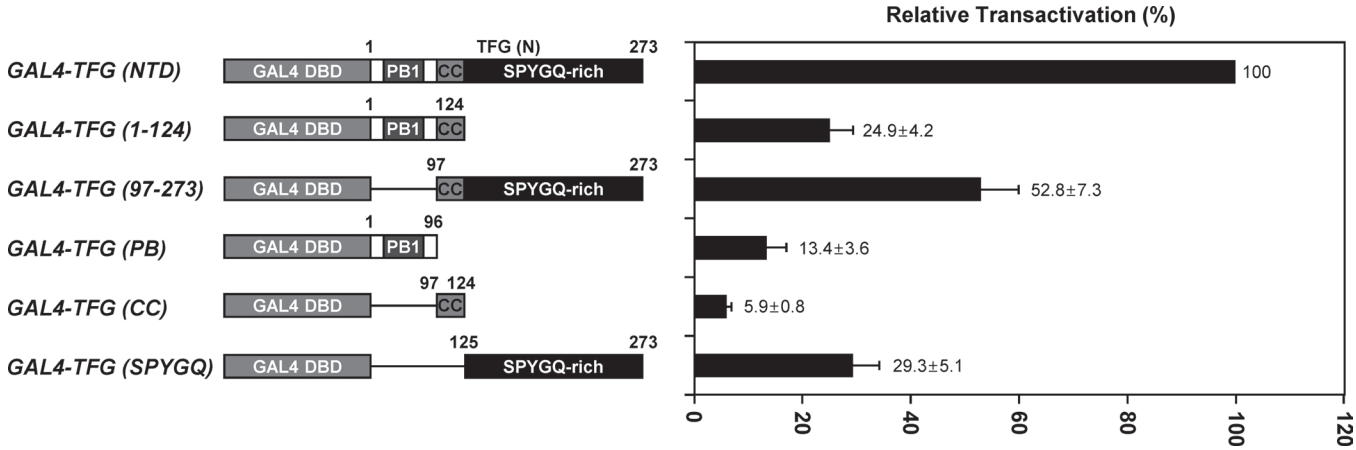

Fig. 7. Functional regions of TFG (NTD). Shown on the left are schematic representations of the deletion constructs of TFG (NTD). Numbers refer to amino acid residues. A reporter plasmid, pG5 luc, was cotransfected into 293T cells with the various GAL4-TFG (NTD) deletion mutants. Relative transcriptional activation values are shown on the right as the mean increases ± SEM relative to a value of 100% for the transfection of the GAL4-TFG (NTD). The results represent the mean of three independent experiments performed in duplicate.

which suggests that the difference in transactivation potential between $hTAF_{II}68$-TEC and TEC may be due to differences in their NTDs. Consistent with this hypothesis, the TFG (NTD) of TFG-TEC was capable of activating transcription when it was tethered to the GAL4 DBD (Figure 6). These results are also consistent with the presence of an activation domain(s) within the TFG (NTD). TFG (NTD) shares sequence homology with both $hTAF_{II}68$ and EWS (7). Hence, TFG (NTD) may stimulate transcription in a manner similar to that proposed for $hTAF_{II}68$ (48), i.e. by playing a role during the conversion of the preinitiation complex from a closed to an open conformation. Additional experiments are needed to explore this possibility.

In conclusion, the findings of the present study provide evidence that *TGF-TEC* is a potential oncogene that may be necessary for tumorigenesis in human EMCs although additional genes may co-operate with *TFG-TEC* or be required for tumor progression. TFG-TEC probably contributes to oncogenesis by modulating key TEC downstream target genes; thus, it would be interesting to determine which downstream target gene is critical for tumorigenesis and whether TFG-TEC collaborates with this/these gene(s) to generate human EMCs. In future experiments, we will focus on identifying critical endogenous targets modulated by TFG-TEC in human EMCs.

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